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Stability evaluation of an immobilized enzyme system for inulin hydrolysis

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Abstract

The stability of free and Amberlite-immobilized inulinase, aiming at inulin hydrolysis was evaluated. The apparent activation energy of the biotransformation decreased when the immobilized biocatalyst was used, suggesting diffusional limitations, despite a decrease in the optimal temperature for catalytic activity for the immobilized biocatalyst. Thermal deactivation, of both forms of the biocatalyst, was evaluated by the linear inverted model. Inulinase immobilization consistently enhanced half-life of the enzyme, which increased up to 6-fold, as compared to the free form. Mean enzymatic activity was computed for both forms of the biocatalyst, and evidenced a decrease of optimal temperature with increased incubation periods. The deactivation energies estimated by an Arrhenius plot, evidenced a decrease of roughly 20% when free inulinase was used. The immobilized biocatalyst was effectively reused in successive batch runs for the hydrolysis of a 5% inulin solution.

Keywords: Enzyme immobilization; Inulin hydrolysis; Inulinase; Thermal stability

1. Introduction

Inulin is a polysaccharide consisting of linear β-2,1 linked polyfructose units (Kaur & Gupta, 2002), terminated by a glucose residue through a sucrose-type linkage at the reducing end (Yun et al., 2000), that can be found in Jerusalem artichoke, dahlia tubers, chicory roots garlic, asparagus root and salsify (Kaur & Gupta, 2002). The use of this polyfructan as raw material for the production of high fructose syrups (Peters & Kerkhoofs, 1983; Vandamme & Derycke, 1983; Wenling, Huiying, & Shiyuan, 1999; Zittan, 1981) or inulooligosaccharides (Cho, Sinha, Park, & Yun, 2001a, 2001b; Yun, Song, Choi, Choi, & Song, 1999; Yun et al., 2000) has been established. The total or partial hydrolysis of inulin, leading to syrups with high fructose content, usually designated ultra-high fructose syrups (UHFS) or to functional sweeteners, respectively, is achieved by the action of exoinulinase (EC 3.2.1.80) (Kulminskaya et al., 2003) or the synergistic action of exoinulinase and endoinulinase (EC 3.2.1.7) (Nakamura, Ogata, Shitara, Nakamura, & Ohta, 1995), if the goal is UHFS; or by the action of endoinulinase, if short chain fructans, namely oligofructose, are aimed at, as a result of partial enzymatic inulin hydrolysis (Kaur & Gupta, 2002; Yun, Kim, Yoon, & Song, 1997a).

Inulinases are fructofuranosyl hydrolases produced by a wide array of microorganisms, comprehending bacteria, fungi and yeast. Among these, the most common sources for inulinases are *Aspergillus* spp. and *Kluyveromyces* spp. (Pendey et al., 1999), alongside with *Pseudomonas* spp. (Kim, Choi, Song, & Yun, 1997; Yun, Kim, Kim, & Song, 1997b), *Xanthomonas* spp. (Park, Bae, You, Kim, & Yun,

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1999), *Penicillium* spp. (Onodera & Shiomi, 1988), *Chrysosporium* spp. (Xiao, Tanida, & Takao, 1989) and *Bacillus* spp. (Zherebtsov, Shelamova, & Abramova, 2002). More recently, the production of recombinant inulinases has also been reported (Park, Jeong, Kim, Yang, & Chae, 2001; Yun et al., 1999; Zhang, Zhao, Zhu, Ohta, & Wang, 2004). Both the products, from total or partial hydrolysis, are commercially relevant.

Currently the commercial production of high fructose syrups, which are used as low caloric sweeteners, given the enhanced sweetness of fructose over sucrose, roughly a 1.3-fold increase in a solids weight basis (http://www.lsbu.ac.uk/biology/enztech/maltose.html, accessed 11 April 2005), relies multi-enzymatic starch hydrolysis, combined with glucose isomerization, and a separation step in a chromatographic column, if a fructose content above 42% is envisaged, to yield high fructose corn syrups, (HFCS) (Huisman & Gray, 2002; Toumi & Engell, 2004). The consumption of HFCS has been rising steadily, as evidenced by the production in the USA, which increased from 2.2 million ton in 1980 to 9.4 million tons in 1999 (Coulston & Johnson, 2002). Lower complexity, higher fructose yield and concomitant cost reduction are easily foreseen for a production process based on inulin hydrolysis. On the other hand, the functional food world market which includes functional sweeteners, such as inulooligosaccharides (or oligo-fructose or fructo-oligosaccharide) (Cho et al., 2001a, Cho, Sinha, Park, & Yun, 2001b; Park et al., 2001), is quite huge and the particular segment embracing these sweeteners is still growing (Holzapfel & Schillinger, 2002). The broad use of oligo-fructose as a food ingredient is related to its acknowledged health benefits, namely its positive interaction with intestinal flora (Alles, Scholtens, & Bindels, 2004; Van Loo et al., 1999), and its role as low caloric sugar replacement in chocolate, ice cream or chewing gum (Park et al., 2001). Furthermore, a single given inulinase based system can be used for either fructose or oligo-fructose production, depending on the selection of the appropriate operational conditions (Nakamura et al., 1995).

The application of enzymes in practical processes often requires biocatalyst immobilization, which enhances thermal stability, allows repeated or continuous use of the biocatalyst and, furthermore eases downstream processing (Schmid et al., 2001; Van Beilen & Li, 2002). Such an approach has been used for inulin hydrolysis to high fructose syrups, using either enzymes (Ettalibi & Baratti, 1992; Gupta, Kaur, Kaur, & Singh, 1992; Kim, Byun, & Uhm, 1982; Nakamura et al., 1995; Peters & Kerkhoofs, 1983; Wenling et al., 1999) or whole cells with inulinase activity (Barranco-Florido, García-Garibay, Gómez-Ruiz, & Azaola, 2001). A hydrolytic system, based in on inexpensive immobilization method, may prove quite useful, particularly if large scale application is foreseen (Katchalski-Katzir & Kraemer, 2000; Schmid et al., 2001). A thorough characterization of a given bioconversion system is necessary in order to evaluate its feasibility. In a previous paper the use of a simple, low-cost methodology (Obón, Castellar, Iborra, & Manjón, 2000) for inulinase immobilization and concomitant application on inulin hydrolysis to fructose was ascertained, and kinetic characterization was performed (Rocha, Catana, Ferreira, Cabral, & Fernandes, 2006). The present work seeks to provide a deeper insight of this bioconversion system based in the immobilization of a commercial inulinase preparation onto Amberlite. In order to do so, the thermal stability of the immobilized enzyme preparation was evaluated and matched to the free form of the biocatalyst. Biocatalyst reuse, in successive batch runs with an initial inulin concentration of 5.0% (w/v), was performed to assess the operational stability of the biotransformation system.

2. Materials and methods

2.1. Biocatalyst

Fructozyme L, a commercial preparation of inulinases from *Aspergillus niger*, was provided by Novozymes.

2.2. Enzyme immobilization

Fructozyme L was immobilized onto Amberlite IRC 50 (Rohm and Haas), as described previously (Rocha et al., 2006).

2.3. Effect of temperature in hydrolytic activity

The biotransformations were started by adding 10 μl of a 10-fold diluted solution of Fructozyme L in 100 mM acetate buffer, pH 5.5, or a given amount of the immobilized biocatalyst, so that similar enzyme concentrations were present, to a 5 g l^{-1} solution of inulin, also in 100 mM acetate buffer, pH 5.5. Bioconversion runs were performed in a temperature range of 30–70 °C, and with a stirring speed of 600 rpm, in order to prevent external mass transfer resistances, as determined in preliminary trials. All trials were performed in duplicate, at least, in a magnetically stirred jacketed glass vessel, of 25 ml volume. Samples (50 μl) were taken periodically, in 1 min gaps up to 5 min, or up to 25 min in 5 min gaps, when the free or immobilized biocatalyst were evaluated, respectively, and immediately assayed for quantification of reducing sugars.

2.4. Thermal stability

A given amount of free or immobilized biocatalyst was incubated in 100 mM acetate buffer, pH 5.5. Briefly, a 5-fold diluted commercial enzyme preparation was incubated at different temperatures. Periodically, 10 μl samples were taken periodically and added to 3 ml of a 5 g l^{-1} solution of inulin in 100 mM acetate buffer, pH 5.5, to quantify residual catalytic activity. For the immobilized biocatalyst, 6 g of Amberlite containing immobilized inulinase were incubated in the buffer solution. Periodically 5 ml samples

of the suspension were taken, and the solid phase recovered by centrifugation (room temperature, 5 min under 1600*g*). Samples of the supernatant were evaluated for protein content. 200 mg of the immobilization matrix were then added to 15 ml of a 5 g l^{-1} solution of inulin in 100 mM acetate buffer, pH 5.5, to quantify residual catalytic activity. Sample collection was performed up to three days. Bioconversion runs were performed at 55 °C with 600 rpm magnetic stirring. All trials were performed in duplicate, at least, in a magnetically stirred jacketed glass vessel, of 25 ml volume.

2.5. Biocatalyst reuse

Immobilized biocatalyst (20 mg) were added to 1.5 ml of a 50 g l^{-1} inulin solution in 100 mM acetate buffer, pH 5.5. Prior to the first biotransformation run, the biocatalyst was thoroughly washed in acetate buffer to remove any loosely bound protein. The biotransformation was performed in a 2.0 ml vessel with 600 rpm magnetic stirring. After a 24 h-run, the reaction was stopped and the immobilized biocatalyst was allowed to settle. The supernatant was removed and assayed for quantification of reducing sugars and free protein. The solid phase was thoroughly washed with acetate buffer and fresh inulin solution was added for a factor biotransformation run. All trials were performed in duplicate.

2.6. Analytical methods

Quantification of reducing sugars was performed by the DNS method (Miller, 1959). Protein was quantified by the Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951).

3. Results and discussion

3.1. Activation energy of inulin hydrolysis

The rate of enzyme-catalyzed reaction increases with temperature up to a limit, then decreases due to denaturation. The activity profile, with temperature, of inulin hydrolysis by inulinase, either immobilized onto Amberlite or in a free form, is given in Fig. 1. Catalytic activities, in the temperature range evaluated were expressed as a percentage of the maximum activity. Trials were performed at pH 5.5, since this was identified as the optimum bulk aqueous phase pH for this bioconversion system using the immobilized biocatalyst (Rocha et al., 2006). A decrease in the optimum temperature of the immobilized inulinase was observed as compared with the free form, an effect that is probably the outcome of changes in physical and chemical properties of the enzyme due to immobilization. Such a decrease in the optimum temperature has been observed when immobilized enzymes are compared with the free form and this effect does not imply lower stability of the immobilized enzyme (Busto, Ortega, & Perez-Mateos, 1997; Geng et al., 2003; Kim et al., 1982; Rogalski, Szczodrak, Pleszczyhka, & Fiedurek, 1997; Woodward, 1985). The Arrhenius plots in a temperature range of 30 °C to the optimum of each form of the biocatalyst were linear (data not shown), suggesting that the Arrhenius expression is a reliable model to depict the effect of temperature on catalytic activity (Akgöl, Kaçar, Denizli, & Arıca, 2001). Activation energies for free and immobilized inulinases were determined as 30.0 and 26.6 kJ mol^{-1}, respectively. Kim et al. (1982) estimated the activation energy in 23.4 kJ mol^{-1} for inulinase from *Kluyveromyces fragilis* no. 351 immobilized on aminoethylcellulose, Bajpal and Margaritis Bajpal and Margaritis (1985) reported activation energies of 26.6 and 9.5 kJ mol^{-1}, respectively, for free and gelatin immobilized *Kluyveromices marxianus* cells with inulinase activity, whereas Ettalibi and Baratti (1992) reported activation energies of 29.9 kJ mol^{-1} for both free and porous glass-immobilized inulinases from *Aspergillus ficuum* (Ettalibi & Baratti, 1990, 1992). The decrease in the apparent activation energy suggests that the bioconversion system currently evaluated is diffusion-controlled rather than kinetically-controlled, when the immobilized inulinase is used, as put forward by Lamb and Stuckey (2000) while assessing the effect of temperature on the activity of free and immobilized β-galactosidase and by El-Masry et al. (2001) while studying immobilization of β-galactosidase onto nylon membranes, which is likely to occur given the bulky inulin molecule, but it is also suggestive of biocatalyst stabilization (Rogalski et al., 1997).

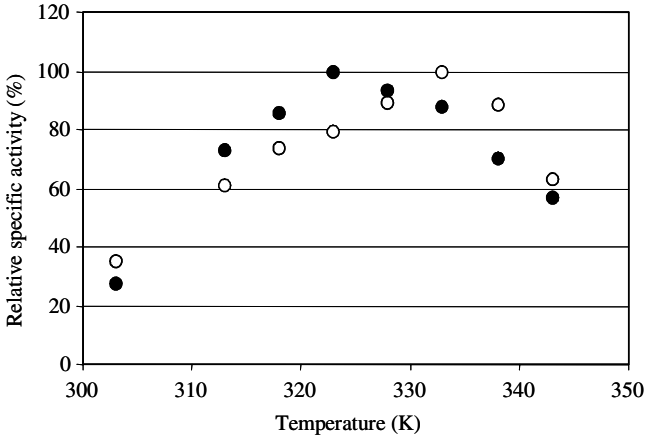

Fig. 1. Effect of the temperature of the biotransformation medium in the biocatalytic activity of free (open dots) and Amberlite-immobilized (closed dots) inulinase. A 5 g l^{-1} inulin solution in a 100 mM acetate buffer pH 5.5 was used. Standard deviation did not exceed 5%.

3.2. Thermal stability of free and immobilized inulinase

The feasibility of biotransformation processes is largely dependent on the maintenance of biocatalytic activity over time. Thermal deactivation of biocatalyst is one of the major causes for activity decay, thus the evaluation of thermal stability of the biocatalyst is a key issue for the effective characterization of a bioconversion system. Incubation of

the free enzyme above 50 °C led to a rather swift loss of catalytic activity, which is typical of most enzymes (Price & Stevens, 1988), whereas a marked enhancement of thermal stability was achieved with enzyme immobilization, since no activity decay was observed after one hour's exposure up to 60 °C (Fig. 2). Bajpal and Margaritis (1985) reported fairly stable inulinase activity of free and immobilized cells of *K. marxianus* up to 50 and 60 °C, respectively, above which a swift decrease of activity was observed, in time-span experiments of 2 h. Enhancement of thermal stability, due to immobilization, has been reported for several enzymes (Akgöl et al., 2001; Alkorta, Garbisu, Llama, & Serra, 1996; Bayramoglu, Akgöl, Bulut, Denizli, & Arıca, 2003; Chang & Juang, 2004), among them inulinases (Bajpal & Margaritis, 1985; Kim et al., 1982; Wenling et al., 1999). Half-lives of both free and immobilized biocatalyst were estimated using the linear inverted model (Cardoso & Emery, 1978), where it is shown that immobilization enhances the half-life of the biocatalyst as much as (roughly) 6-fold (depending on the temperature) as compared to the free form (Table 1). A half-life of roughly 4 h at 50 °C was observed for inulinase on aminoethylcellulose (Kim et al., 1982), while Gupta et al. (1992) reported a half-life of 45 min for inulinases of *Fusarium oxysporum* immobilized in DEAE-cellulose at 50 °C (Gupta et al., 1992), in both cases with biocatalyst incubated in buffer, as in the present system. Kim et al. (1982) evidenced the stabilizing effect of the substrate, since no activity decay was observed after 4 h when inulinase immobilized on aminoethylcellulose was incubated in the presence of a 7% (w/v) inulin solution in acetate buffer (Kim et al., 1982). Hence a half-life of 13.9 days was estimated for this immobilized enzyme in the presence of a 7% (w/v) inulin solution and Ettalibi and Baratti (1992) estimated a half-life of 17.9 days for porous glass immobilized inulinases in the presence of a 5% (w/v) inulin solution (Ettalibi & Baratti, 1992), in both cases working with packed bed column reactor operating in continuous mode at 40 °C. The calculated half-life of 21 days at this same temperature in the currently described thermal stability studies is therefore quite promising. Exposure of an enzyme to a given temperature leads to catalytic decay over time, a feature that is intensified with a temperature increase.

Table 1
Half-life of free and immobilized inulinase

T (K)	Half-life, free form (days)	Half-life, immobilized form (days)
303	1.9×10^{1}	8.8×10^{1}
313	4.1×10^{0}	2.1×10^{1}
323	1.4×10^{0}	5.6×10^{0}
328	2.2×10^{-1}	1.1×10^{0}
333	7.8×10^{-1}	5.9×10^{-1}
343	6.7×10^{-2}	1.3×10^{-1}

Trials were performed in 100 mM acetate buffer, pH 5.5, at different temperatures.

An effective biocatalyst system must combine high catalytic activity and high thermal stability, parameters that are often at their best at different temperatures. A compromise between high catalytic activity and long term stability is then necessary, which can be established through the mean enzymatic activity, E_m (Cabral, Kennedy, & Novais, 1982);

$$E_m = \frac{\int_{t_0}^{t_t} E(t)\mathrm{d}t}{t_t - t_0}, \text{ and in this particular case}$$

$$E(t) = \frac{E_i}{1 + k_d t},$$

where E_i is the initial activity, t_t is the final time of operation, t_i is the initial time of operation, t is the time of operation and k_d is the decay constant. Values for E_m were computed by integration over a 72 h period (Figs. 3 and 4), when it evident that longer incubation periods lead to a shift in the optimal temperature.

The values of the deactivation kinetics for each temperature were used to determine the activation energy for deactivation of the enzyme (E_d), that was calculated assuming that the Arrhenius equation, $k_d = A \exp(-E_d R^{-1} T^{-1})$,

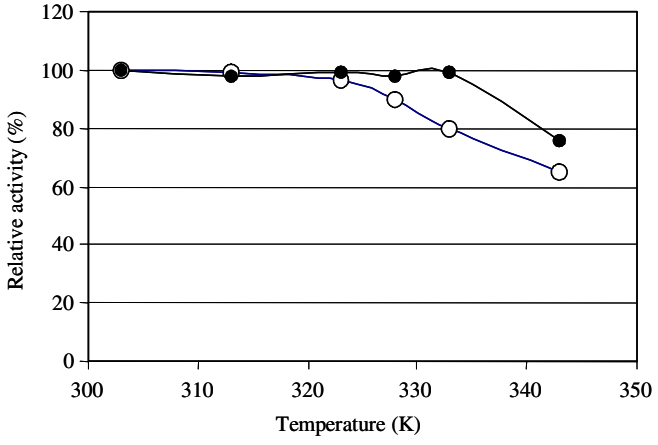

Fig. 2. Thermal stability free (open dots) and Amberlite-immobilized (closed dots) inulinase after a 1 h incubation at different temperatures measured at pH 5.5. Standard deviation did not exceed 5%.

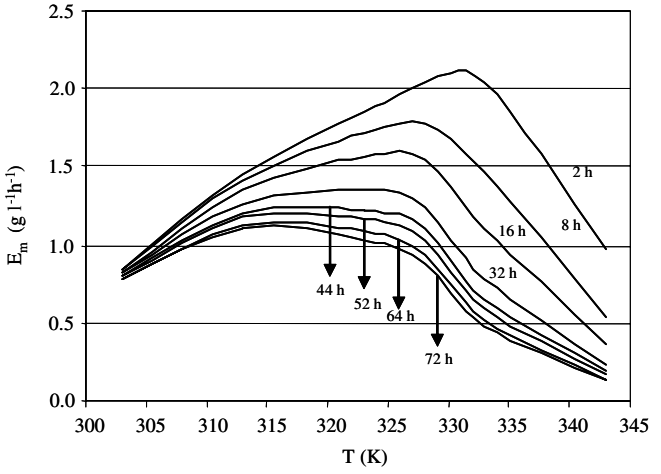

Fig. 3. Mean enzymatic activity of free inulinase. Computed values for different incubation periods are depicted.

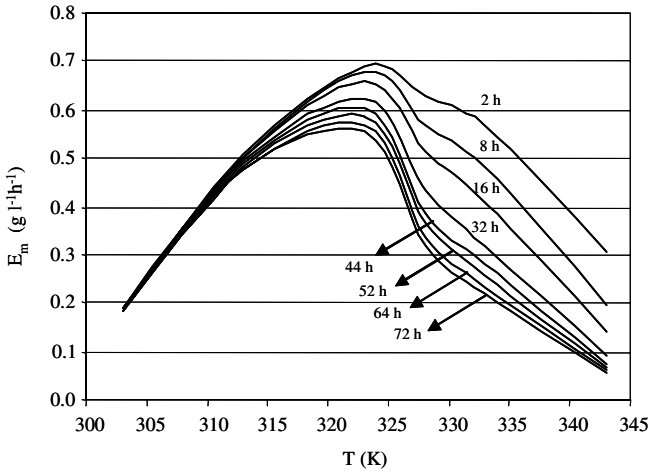

Fig. 4. Mean enzymatic activity of Amberlite-immobilized inulinase. Computed values for different incubation periods are depicted.

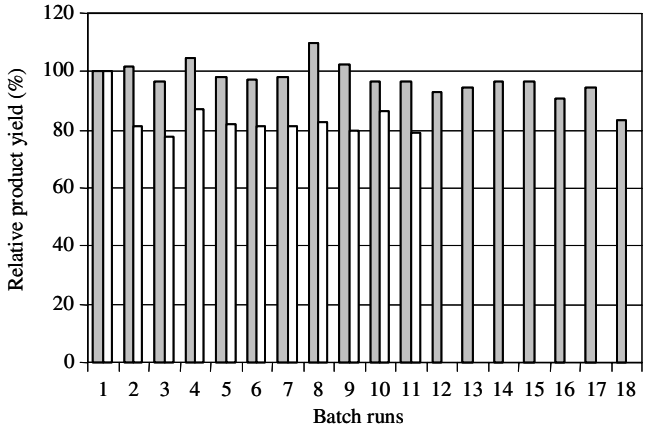

Fig. 6. Effect on the final product yield, of the repeated use of Amberlite-immobilized inulinase for the hydrolysis of a 5% (w/v) inulin solution. Biotransformation batch runs (24 h) were performed at 40 °C (grey bars) and 50 °C (white bars) at pH 5.5. Standard deviation did not exceed 10%.

was valid for the system evaluated (Lamb & Stuckey, 2000; Naidu & Panda, 2003).

The natural logarithms of the data were plotted and since they lay along a straight line (Fig. 5), it can be concluded that the Arrhenius law adequately described the temperature-dependence of both free and immobilized biocatalysts.

Given data from Fig. 5, values for the deactivation energy and pre-exponential constants were estimated as $E_d = 144$ kJ mol^{-1} and $\ln A = 49.4$, for immobilized inulinase and $E_d = 121$ kJ mol^{-1} and $\ln A = 41.8$, for the free enzyme. The reduction in E_d further suggests that the free enzyme form is less stable than the immobilized enzyme (Rha et al., 2005; Rodriguez-Nogales & Delgadillo, 2005).

3.3. Biocatalyst reuse

Immobilized biocatalyst was used repeatedly in several batch hydrolysis runs at 40 and 50 °C (Fig. 6). No decay in product yield after 24 h biotransformation was observed in trials performed at 40 °C, and only a decrease of roughly 20% was observed for trials performed at 50 °C, which apparently confirms the stabilizing effect of the substrate. The experimental data gathered also highlight mechanical stability of the immobilization matrix with prolonged exposure to stirring.

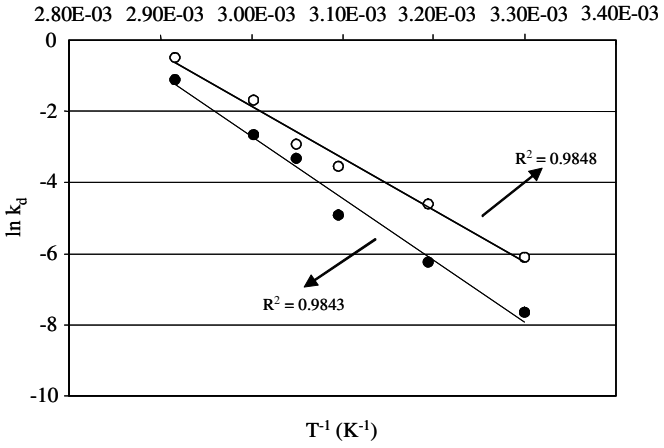

Fig. 5. Arrhenius plot of deactivation rate constant, of free (open dots) and Amberlite-immobilized (closed dots) inulinase.

4. Conclusions

Activation energies for the hydrolysis of inulin with free and Amberlite-immobilized inulinase were determined. The lower value observed for the immobilized system is suggestive of diffusional limitations but also of enzyme stabilization, the latter being confirmed by the longer half-lives at different temperatures and, concomitantly, higher deactivation energy, of the immobilized form. The data given also highlight that the temperature profile for initial biocatalytic activity is different from the thermal stability profile, a behaviour often observed within (bio)catalytic systems, where the optimal temperature shifts to lower values with increased incubation periods. The immobilized biocatalyst was successfully reused in repeated biotransformation runs with no significant decrease in the final product yield, suggestive of adequate operational and mechanical stability, making it a promising candidate for the development of a highly effective set-up for the production of high fructose syrups from inulinase.

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